*GENETICS OF SOMATIC MAMMALIAN CELLS, VII. INDUCTION AND ISOLATION OF NUTRITIONAL MUTANTS IN CHINESE HAMSTER CELLS**

BY FA-TEN KAO AND THEODORE T. PUCK†

DEPARTMENT OF BIOPHYSICS, UNIVERSITY OF COLORADO MEDICAL CENTER, DENVER

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In a previous paper[1] a method was suggested for isolation of nutritionally deficient mutants in mammalian cells (Fig. 1). A large cell population is exposed to 5-bromodeoxyuridine (BUdR) in a medium lacking certain nutrilites. Those cells competent to grow in the given medium incorporate BUdR into their DNA and are subsequently killed by an exposure to near-visible light. The deficient mutants do not incorporate the brominated analogue and are unaffected by the illumination. These are then grown up into colonies by replacement of the

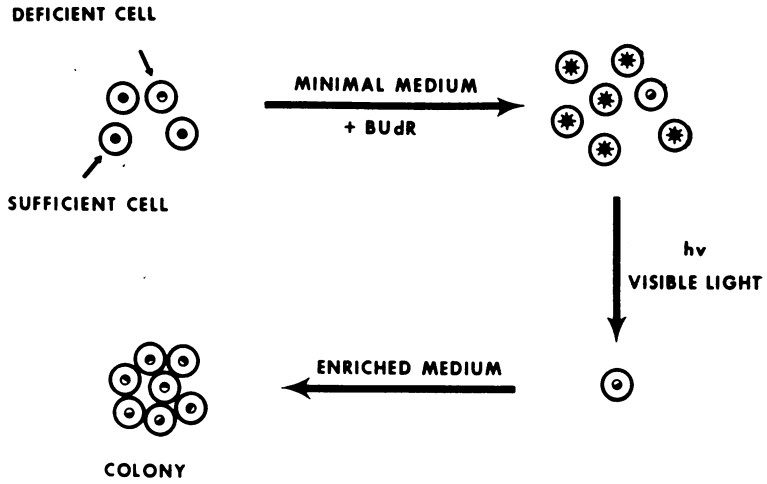

FIG. 1.—Schematic representation of the BUdR-visible light technique for isolation of nutritionally deficient mutant clones. The mixed cell population is exposed to BUdR in a deficient medium in which only the prototrophs can grow. These alone incorporate BUdR into their DNA and are killed on subsequent exposure to a standard fluorescent lamp. The medium is then changed to a composition lacking BUdR and enriched with various nutrilites, and the deficient cells grow up into colonies. About 10^{-4} of the slowly growing prototrophs may also escape killing by this method, so each colony is tested for its ability to grow in enriched but not in deficient medium.

nutritionally deficient medium with one enriched with various metabolites. The resulting colonies can be isolated and their nutritional requirements determined. The present report describes application of this method to the induction and isolation of mutants of Chinese hamster cells.

Materials and Methods.—The parental cell from which all the cultures described here were derived is the proline-requiring, Chinese hamster ovary cell, CHO/Pro$^-$, which has only 21 chromosomes.[2] In some experiments, the subclone, CHO/Pro$^-$ Kl, which possesses a stemline of only 20 chromosomes, was utilized. F12 medium[3] was used for routine cell cultivation and single-cell plating,[4] except where a deficient form of this medium was employed, as described. All plates were supplemented either with 10% fetal calf serum,

or, when the introduction of small-molecular-weight nutrilites was to be avoided, with an equivalent amount of the macromolecular fraction alone, as described in earlier reports.[1, 2] The treatment with BUdR and visible light was carried out as described previously. After treatment in a minimal medium with BUdR, cells were illuminated with a standard fluorescent lamp, and then reincubated in an enriched medium consisting of complete F12 plus 10% whole fetal calf serum. This procedure results in colony formation from only 10^{-4} of the nutritionally sufficient cells, and from approximately half of the cells that have a nutritional deficiency like that of the proline-requiring mutant.[1] Consequently the practice was adopted of exposing about 10^6 cells to the BUdR-visible light procedure, and of testing approximately 100 of the colonies developing in the enriched medium for their ability to grow in a deficient medium.

Experimental Results.—(1) *Preparation of a minimal growth medium:* The standard F12 medium was originally developed to produce maximal growth rates for Chinese hamster cells in the absence of protein and consequently contains nutrilites that do not materially affect the growth rate or plating efficiency under the conditions employed here. Experiments were carried out to determine the minimal nutrilites required to produce maximal growth rate and plating efficiency for the standard CHO cell when supplemented with the macromolecular fraction of fetal calf serum. It was found that the following components could be omitted from F12 without affecting growth significantly: glycine, alanine, aspartic acid, glutamic acid, thymidine, hypoxanthine, inositol, vitamin B_{12}, and lipoic acid. The minimal medium obtained by omission of the nine indicated metabolites is called F12D.

(2) *Spontaneous mutation to auxotrophy:* Tests were carried out to determine whether spontaneous mutation in an appreciable frequency occurs at any of the loci responsible for nutritional independence with respect to the nine metabolites omitted from F12. No such spontaneous mutations were found when 10^6 cells of each clone were examined by the BUdR-visible light technique (Table 1). If the efficiency of detection of proline-deficient mutants in the BUdR-visible light procedure also holds for the nine metabolites investigated here, the individual frequencies of spontaneous mutation to deficiency for any one of these metabolites would appear to be $\leqq 2 \times 10^{-1}$.

TABLE 1. *Yield of deficient mutants obtained in separate experiments with the two mutagens and the two standard clones employed.*

Mutagen	Cell employed	No. of clones surviving treatment with BUdR + near-visible light	No. of clones tested	No. of deficient mutant clones found
None	CHO/Pro$^-$	129	100	0
None	Kl subclone	125	98	0
MNNG	CHO/Pro$^-$	160	100	1
MNNG	Kl subclone	172	84	17
EMS	Kl subclone	175	91	8
EMS	Kl subclone	198	84	21

The MNNG was employed at a concentration of 0.50 μg/ml for 16 hr, which leaves about 26% of the original cell population viable; while the EMS was used in a concentration of 200 μg/ml for 16 hr, which leaves 78% of the cells viable. After exposure to the mutagen, 10^6 surviving cells were subjected to the BUdR-visible light technique, the colony survivors were counted, and approximately 100 of these tested for ability to grow in F12 but not in F12D.

(3) *Mutagenesis to auxotrophy by ethyl methanesulfonate (EMS) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)*: Single cell survival curves were first constructed for each of these compounds, whose effectiveness in producing gene mutations in bacterial systems is well recognized.[5] Two hundred cells were inoculated into each of a series of Petri dishes in F12 supplemented with 10 per cent fetal calf serum. After six hours of incubation, EMS (Eastman Organic Chemicals, Rochester, N.Y.) or MNNG (Aldrich Chemical Co., Milwaukee, Wis.) was added in an appropriate concentration, and incubation was continued for an additional 16 hours, which is slightly more than one generation period. Thereafter, the medium was removed, the cells were washed three times, fresh growth medium was added, and incubation was continued for another five to seven days, after which the surviving colonies were counted. The results shown in Figure 2 demonstrate that, whereas the survival curve for EMS treatment has a large initial shoulder, the survival curve for MNNG approximates a simple exponential relationship with a much higher cell toxicity than that of the other mutagen.

Mutagenesis experiments were performed with CHO/Pro$^-$ and its K1 subclone. In each experiment approximately 2–4 $\times$ 10^6 cells were treated for 16 hours with 200 μg/ml of EMS or with 0.50 μg/ml of MNNG, conditions providing the largest concentration of each drug at which a reasonably high cell survival is still achieved (78 and 26% for the EMS and MNNG, respectively). After this period, the medium was removed, the cells washed, and fresh growth medium re-

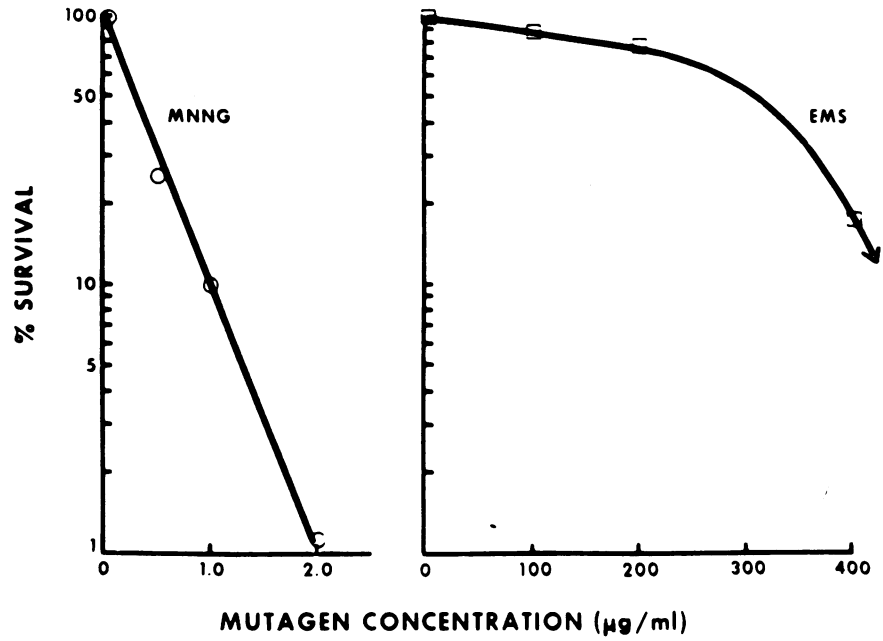

FIG. 2.—Survival curves for Chinese hamster cells exposed for 16 hr at 37°C to each of the mutagens of this study. Both cell clones utilized responded in similar fashion. The arrow in the EMS curve represents the fact that at 600 μg/ml of EMS, the plating efficiency was zero.

placed. The cells were grown for an additional six days in order to allow full development of any end-point mutations. The cells were trypsinized and 10^6 survivors were distributed among 40 plates, each finally containing 2.5×10^4 viable cells, and were then treated with BUdR and near-visible light in standard fashion for isolation of deficient mutants. The results are summarized in Table 1, and indicate effective mutagenesis from both agents.

(4) *Analysis of the induced nutritional requirements:* The new nutritional requirements for 44 mutant clones here isolated are shown in Table 2. Two

TABLE 2. *Nutritional supplements required by the various newly isolated deficient mutants.*

Clone from which mutant originated	Mutagen employed	No. of Mutants Found with the Indicated Nutritional Deficiencies			
		Glycine	Glycine + thymidine + hypoxanthine	Inositol	Other requirements
CHO/Pro$^-$	MNNG	1	0	0	0
Kl subclone	MNNG	4	10	1	2
Kl subclone	EMS	27	2	0	0

All mutants grow with plating efficiencies approximately 100% and zero in F12 and F12D, respectively.

clones have not yet been completely analyzed but appear to have different requirements from the others. All clones display a plating efficiency of zero in F12D medium and virtually 100 per cent when the medium is supplemented with the agents shown. All clones possess, in addition, the proline requirement described earlier that existed in the original cell culture from which these clones were derived.

Further measurements on these mutants are in progress. The response to glycine by one of the mutants here isolated is shown in Figure 3 and a curve demonstrating the effect of varying glycine concentrations is shown in Figure 4. Spontaneous reversion to glycine independence for this mutant was sought, but no revertants have yet been found in a test population of 2×10^7 cells. In another glycine-requiring mutant, one revertant was found in a test population of 5×10^7 cells. Hence the revertant frequencies for these particular mutants appear to lie in the neighborhood of 3×10^{-8}, a value which permits good resolution in genetic experiments.

Mutagenesis with these agents appears to be unaccompanied by the extensive chromosomal alterations characteristic of the action of mutagens like X rays on mammalian cells. Thus karyotypic analysis of five of the deficient mutants here described have revealed no detectable changes in number or structure of the chromosomes, as compared with the parental cell.

(5) *Mutagenesis to prototophy at the proline locus:* Both mutagens were also tested for their ability to produce revertants to proline-independence in the CHO/Pro$^-$ cell. In this case it was necessary only to plate the cells in a proline-deficient medium after treatment with the mutagenic agent, and to count the colonies which appear. The results of five experiments have been pooled in Table 3, comparing the effectiveness of each mutagen. EMS appears to be more effective than MNNG as a mutagen for this particular locus.

Discussion.—These experiments demonstrate that mutagenesis by agents

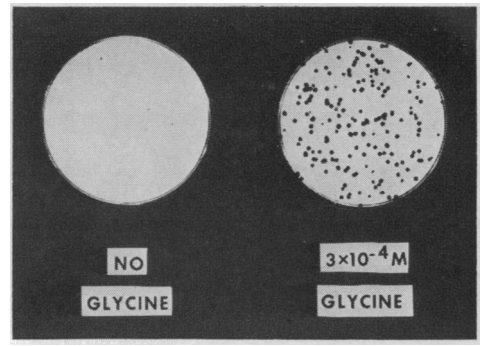

FIG. 3.—Demonstration that the glycine-requiring mutants induced by the chemical agents here employed yield no colonies in the absence of glycine but 100% plating efficiency in its presence. The prototroph has 100% plating efficiency in either case.

known to produce single gene mutations in bacteria and isolation of the resulting nutritionally deficient or sufficient forms can be carried out in simple fashion in mammalian cells. The techniques appear to permit production and quantitation of mutations to auxotrophy as well as to prototrophy.

The frequent appearance of the requirement for glycine in auxotrophic cells isolated after treatment with EMS and MNNG is noteworthy. In previous papers we have discussed the need for cells with specific chromosomal monosomies to overcome the difficulties of genetic analysis with diploid cells.[2, 6] In the present experiments one of the glycine mutants originated in the 21-chromosome CHO/Pro$^-$ cell, while all of the remainder (including the mutant with the hypoxanthine and the thymidine requirements) arose from the 20-chromosome, K1 subclone. However, analysis revealed that even the glycine-deficient mutant from CHO/Pro$^-$ possesses only 20 chromosomes.[6a]

As a working hypothesis, we presume that genes necessary for glycine biosynthesis are contained in one of the missing chromosomes of the cells under study, so that mutation of such loci on the remaining homologous chromosome resulted in expression of the glycine deficiency. One possible explanation for the high

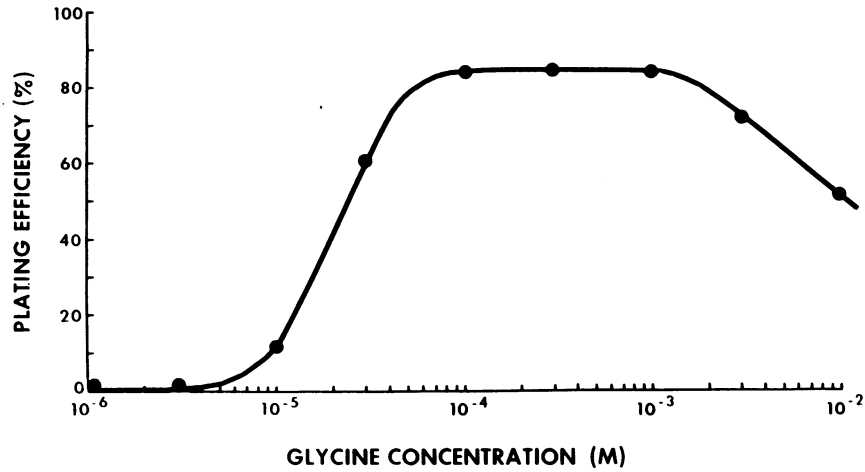

FIG. 4.—Dose-response curve to glycine by one of the glycine-requiring mutants of this study.

TABLE 3. *Test of EMS and MNNG to produce reversion to proline independence of the CHO/Pro⁻ cell.*

Mutagen	Total no. viable cells tested ($\times 10^6$)	No. of revertants	Reversion frequency ($\times 10^{-6}$)
None	15.2	18	1.2
EMS (200 μg/ml for 16 hr)	9.2	49	5.3
MNNG (0.5 μg/ml for 16 hr)	5.6	16	2.9

frequency of recurrence of the glycine deficiency may be that glycine is the only member of the nine metabolites searched for whose biosynthetic genes lay on the missing chromosomal members. This hypothesis will be tested by repeating these studies on Chinese hamster cells with no detectable chromosomal deficiencies and no heterozygosity. These conditions should be ascertainable by careful chromosomal analysis and by use of cells from the Yerganian Chinese hamsters which have been inbred for long periods so as to achieve a homozygous condition in the overwhelming majority to their genes.[7]

A systematic method which might provide large numbers of chromosomal monosomies in Chinese hamster cells and so permit extension of these studies will be described elsewhere.[8] An alternative possible approach to biochemical genetic studies on such cell cultures, utilizing specific antimetabolites that should greatly increase the number of usable mutations, has also been presented.[6]

The biochemistry underlying the glycine-deficiency mutation remains to be determined. Glycine is usually not required by mammalian cells, although such a requirement has been described in a primary monkey kidney culture.[9] Serine is the major source of glycine in mammalian cells, and the conversion is accomplished by the enzyme serine hydroxymethylase.[10] The one glycine-requiring mutant so far tested failed to utilize increased amounts of serine (10^{-4} to 10^{-1} M) to substitute for glycine. It is possible then that the new deficiency may reflect a serine hydroxymethylase defect.

The conversion of serine to glycine in mammalian cells requires reduced folic acid as a coenzyme, and the one-carbon moiety removed from serine is utilized in pyrimidine synthesis according to the following scheme:[11]

$$\text{Folic acid} \xrightarrow[\text{Folic acid reductase}]{} \text{Tetrahydrofolate (THF)},$$

$$\text{THF} + \text{serine} \xrightarrow[\text{Serine hydroxymethylase}]{} \text{Glycine} + N^5, N^{10}\text{-methylene THF},$$

$$N^5,N^{10}\text{-methylene THF} + \text{dUMP} \xrightarrow[\text{Thymidylate synthetase}]{} \text{Thymidine monophosphate} + \text{dihydrofolate}.$$

The THF is also needed for the eventual production of inosinic acid, which, however, can be formed from hypoxanthine if it is supplied in the medium. Therefore, the mutants which require glycine, thymidine, and hypoxanthine for growth may represent deficiencies in folic acid reductase, so that all three metabolites become necessary. This provisional interpretation is supported by the fact that amethopterin, which inhibits folic acid reductase in mammalian cells, has also been found to introduce a requirement for these same three metabolites.[12]

Test for identity of the various glycine deficiencies may be possible by means of

complementation studies with the use of the heterokaryon-production and cell-fusion techniques.[13, 14] The low frequency of spontaneous reversion of the glycine-requiring mutants here described also makes these cells admirably suited for study of viral transduction and DNA transformation, and for quantitation of mutagenesis by drugs, radiation, and other agents.

The difference in the lethal activity displayed by the two mutagens here studied is noteworthy, and indicates important differences in their mode of action that may be susceptible to further biochemical analysis in this cell system. Studies are continuing, directed at the collection of further mutants, quantitation of mutagenic action by various agents, analysis of the underlying biochemistry of the genetic markers produced, and exploration of the relationships between a variety of mutagenic and carcinogenic agents on mammalian cells.

Summary.—Mutagenesis in Chinese hamster cells by ethyl methanesulfonate and N-methyl-N′-nitro-N-nitrosoguanidine, agents that produce single gene mutations in microorganisms, is described. Auxotrophic mutants deficient in glycine, glycine + thymidine + hypoxanthine, and inositol were isolated by means of the technique that destroys prototrophs by exposure to BUdR followed by illumination with near-visible light. Mutants so obtained are stable and exhibit low reversion rates so that they are useful for many kinds of genetic experiments. Mutation to prototrophy at the proline locus was also achieved with these mutagens. Application of these findings to various problems in mammalian cellular genetics has been indicated.

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† American Cancer Society Research Professor.

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[6a] Analysis of the karyotypes of these clones will be reported elsewhere. However, such analysis is less illuminating than one could hope because the original progenitor CHO/Pro$^-$ has undergone several aberrations which make difficult the identification of the missing parts of the chromosomal complement in it and its subclones.

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